Increased Survival In Puppies Affected By Canine Parvovirus Type II Using An Immunomodulator As A Therapeutic Aid

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Research Article

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Abstract

Background: The canine parvoviral enteritis (CPE) promotes sepsis and systemic inflammatory response syndrome (SIRS). Mortality in this disease is usually registered within 48–72 h post-hospitalization, the critical period of the disease. It has been recently described that the use of an immunomodulator whose major component is monomeric ubiquitin (mUb) without the last two glycine residues (UbΔGG) in pediatric patients with sepsis improves survival. It is known that CXCR4 is the cell receptor of extracellular ubiquitin in humans. The aim of this work was to explore the effect of one immunomodulator (hDLE) as a therapeutic auxiliary in CPE puppies with sepsis and SIRS.

Results: We studied two groups of puppies with CPE confirmed by polymerase chain reaction. The first group received conventional treatment (CT) and vehicle (V), while the second group received CT plus an immunomodulator (I). In both groups, we assessed the survival, clinical condition, number of erythrocytes, neutrophils, and lymphocytes, and values of hematocrit, hemoglobin, plasma proteins, cortisol, and norepinephrine epinephrine, and serotonin, during hospitalization.

Puppies treated with CT+I showed 81% survival and milder clinical signs and a significant decrease in circulating neutrophils and lymphocytes in the critical period of the treatment. In contrast, the CT+V group presented a survival of 42%, severe clinical status, and no improvement of the parameters evaluated in the critical period of the disease. We determined *in silico* that human UbΔGG can stimulate dog CXCR4.

Conclusions: The administration of an immunomodulator (5 mg/day x 5 days) to puppies with CPE under 6 months of age reduces the severity of clinical signs, increases survival, and modulates inflammatory cell parameters. Further studies are necessary to take full advantage of these clinical findings which might be mediated by the human UbΔGG to canine CXCR4 interaction.

Introduction

The causal agent of canine parvoviral enteritis (CPE) is Canine Parvovirus Type 2 (CPV-2), a lytic virus of the *Parvoviridae* family [1]. CPV-2 is an icosahedral, non-enveloped virus; it has a negative-sense single-stranded genome and a molecular weight of 5.5–6.2 kDa [2]. It can affect dogs of any breed, sex, and age, but those under 6 months show higher incidence. The morbidity of the virus is 100%, while mortality among puppies without medical treatment reaches 91%, which can be reduced with veterinary medical care [2, 3].

This parvovirus presents in two ways: viral myocarditis limited to neonatal infections in puppies of non-vaccinated mothers and CPE characterized by a severe gastroenteritis [3] with mucoid or hemorrhagic diarrhea, vomit, depression, dehydration, prolonged capillary refill time [4], abdominal pain, hypo- and hyperthermia [2], and, under severe conditions, hypovolemic shock [3]. The hematological and biochemical evaluation of this disease is characterized by lymphopenia, neutropenia [5], hyperazotemia, hypoalbuminemia, metabolic acidosis or alkalosis, and alterations in coagulation [3].

The treatment of CPE is based on restoring the water balance, correcting metabolic alterations, and preventing secondary bacterial infections [4, 6]. Some clinical studies suggest the use of omega interferon [7], a soluble transferrin receptor (CPV-2 receptor) [8], antiviral drugs [9, 10], and hyperimmune serum [11] as treatments aimed at reducing the viral load instead of solving the sepsis and the systemic inflammatory response syndrome (SIRS), critical elements linked to a higher mortality rate of CPE [4]. Therefore, finding effective treatments to control them is a therapeutic alternative.

Physiopathological alterations of CPE, such as secondary bacterial translocation, destruction of intestinal crypts, neutropenia and immunosuppression due to thymus atrophy, cause sepsis and SIRS in puppies [3, 12]. They exacerbate clinical signs and lead to multiple organ dysfunction syndrome (MODS) and death [12]. There is a high incidence of sepsis and SIRS in CPE patients and thus this is a sepsis model for humans [13].

Additionally, there is a recent report on increased survival of pediatric human patients with sepsis treated with the conventional treatment and an immunomodulator (human dialyzable leukocyte extract-hDLE). The treatment was linked to reduced serum concentration of C-reactive protein (CRP) and circulating neutrophils 72 h post-admission as well as increased lymphocyte count [14]. The active ingredient in the immunomodulator is a mixture of low molecular weight peptides (< 10 kDa), the major being monomeric human ubiquitin (mUb) and a variant with the last two glycine residues deleted (UbΔGG) [15]. Highly conserved among mammals [16], Ub has a low molecular weight and controls inflammation in sepsis when it is extracellular [17], likely through the activation of CXCR4 specially those associated with the vagal afferences [15, 18]. It is possibility induces the activation of the vagal anti-inflammatory arch by modulating the peripheral inflammatory response and blood cortisol levels [19, 20].

The aim of this work was to explore the effect of one immunomodulator (hDLE) as a therapeutic auxiliary in CPE puppies with sepsis and SIRS. For this purpose, we evaluated the clinical symptoms of the disease, survival, numerical changes in neutrophils and lymphocytes counts, and cortisol concentrations as improvement parameters. In addition, we determine the concentrations of norepinephrine (NE), epinephrine (EP), and serotonin (SE) to infer the activity of the vagal tone, and we explored the possible molecular interaction causative of these effects.

Results

Description of study population

The age, sex, breed, and vaccination status of all participants as well as the treatment received are described in Table 1. The age (months) of the patients in the CT + V group was 3.1 ± 0.9 and that of subjects in CT + I, 3.7 ± 1.1. The statistical analysis showed no age differences between groups. The study population consisted of 45% mixed-breed patients and 55% pure-breed patients. In the sample analyzed, only 4/18 of the patients showed vaccination history (attenuated, live virus) at two months of age. The CT administered to the patients can be found in Table 2.

Table 1
Demographic characteristics of the study population. The number of patients per group, age, sex, breed, and vaccination status are described.

Group	Patient	Age (months)	Sex	Breed	Vaccination status
CT + V	1	4	Male	Mixed	No
	2	2	Male	Pit Bull	No
	3	2	Male	Chihuahua	No
	4	4	Male	Blood Hound	No
	5	4	Male	Schnauzer	No
	6	3	Male	Mixed	No
	7	3	Male	Poodle	Yes
CT + I	1	3	Female	Mixed	No
	2	2	Female	Mixed	No
	3	4	Male	Mixed	Yes
	4	2	Female	Pomeranian	No
	5	5	Male	Schnauzer	No
	6	4	Female	Pit Bull	No
	7	3	Female	Mixed	No
	8	5	Male	Mixed	No
	9	4	Female	Schnauzer	Yes
	10	4	Male	Schnauzer	Yes
	11	5	Male	Mixed	No

CT = conventional treatment; I = immunomodulator; V = vehicle

Table 2. Conventional treatment in CT+V and CT+I groups. Treatments were classified according to their action mechanism. The number of patients that received each of the treatments is specified.

Treatment classification	CT+V		CT+I	
Fluid therapy	0.9% NaCl solution	7/7	0.9% NaCl solution	11/11
Antibiotics	Ampicilin	3/7	Ampicilin	5/11
	Metronidazole	5/7	Metronidazole	8/11
	Enrofloxacin	2/7	Enrofloxacin	2/11
	Cephalexin	5/7	Clindamicyn	1/11
			Sulfa-trimethoprim	4/11
Antiemetics	Maropitant citatre	7/7	Maropitant citrate	9/11
	Metoclopramide	4/7	Metoclopramide	5/11
Antiespasmodics	Prifinial	1/7	Butylhioscine	5/11
Gastric mucosa protectors	Ranitidine	4/7	Ranitidine	8/11
	Omeprazole	7/7	Omeprazole	9/11
			Sucralfate	4/11
Analgesics	Buprenorphine	7/7	Buprenorphine	10/11
	Meloxicam	1/7	Lidocaine	5/11
			Metamizole	3/11
Others	0.5% Dextrose	5/7	0.5% Dextrose	8/11
	Aminolyte	2/7		

CT= conventional treatment; I= immunomodulator; V= vehicle.

Confirmation of infection

All patients were confirmed as positive for CPV-2 by end-point PCR (see Figure S3 of supplementary information), because the corresponding region was amplified.

Survival, clinimetric evaluation, weight, and days of hospitalization

The survival results of CT + V *vs* CT + I are represented in Fig. 1, showing a significant difference in the outcome depending on the therapeutic management ($P \leq 0.043$). This suggests that one or some of the components of the immunomodulator are responsible of the improvement of patients' survival. In all the cases, regardless of the treatment, mortality was observed between the first and third day of hospitalization.

The clinical evaluation used in this study aimed at identifying whether the immunomodulator could reduce the severity of the clinical presentation in CPE patients. Regarding the clinical follow-up of the

study groups, a high score indicated that the clinical signs were more severe while a low score showed the severity of the disease was less intense. The statistical comparison between treatments showed significant differences ($H = 19.99$, $d = 90,1$; $P \leq 0.018$). The CT + I group (Fig. 2B) showed less severe clinical signs than CT + V patients (Fig. 2A). The decrease in the clinical score of CT + I was observed since the first day of hospitalization and continued in later days, showing values under basal measurement. Figure 2 shows the clinical score on the different evaluation days per patient and group. In all the cases, the score of patients that died was 41.

The CT + V group showed a reduction in clinical signs only on day 1 of hospitalization and there was an increase on day 2, reaching a value above basal measurement in later days. Since mortality in both groups was observed between days 1 and 3, the size of the CT + V sample was considerably reduced. Then, we analyzed the data obtained from the survivors of each group on days 1 and 2 and detected no significant differences ($H = 3.827$, $d = 30,1$; $P \geq 0.280$). Although the surviving CT + I patients showed lower scores than those in the CT + V group, the scores were 8.55 ± 3.32 on day 1 and 6.89 ± 3.33 on day 2 on the group TC + I, while those of CT + V were on day 2, 10. 00 ± 2.68 on day 1 and 9.67 ± 7.41 on day 2.

Figure 2 depicts the patients that died in the CT + V group exhibited a higher clinical score before death when compared against dead patients of the CT + I group. This suggests that the treatment plus the immunomodulator induced a clinical improvement, which was reflected in mortality, and that the CT + I patients that survived the critical period had a better clinical condition.

When analyzing the weight of the participants by treatment and time, we detected no significant differences in the comparison between change percentages of the treatments (data not plotted). However, CT + I patients showed a positive percentage of weight change at all times evaluated than to the CT + V patients, who showed a negative percentage of weight change. For instance, on the critical day of the disease (day 2) the percentage of weight change in the CT + I group was 1.22 ± 4.90, while in the CT + V group was − 3.06 ± 5.50, at the end of the study the CT + I group showed a percentage of 1.85 ± 13.61 and the CT + V group was of -7.72 ± 9.48. A negative change in weight indicates the patients lost weight with respect to the initial value; in contrast, a positive value is interpreted as weight gain. Considering this, the percentage values of weight change show that the infection with CPV-2 induces weight loss but the administration of the immunomodulator hampers it.

The number of hospitalization days in the surviving CT + I was 6.10 ± 2.26 and 7.00 ± 1.00 in CT + V; we found no significant differences.

Hematological parameters

Hematocrit, hemoglobin, and erythrocyte values showed no significant differences at the beginning of the treatments (data not plotted). The comparison between treatments along the follow-up showed significant differences in hemoglobin levels ($H = 18.18$, $d = 82, 1$; $P \leq 0.030$); CT + V showed a

concentration (g/L) of 104.40 ± 16.83, lower than that of CT + I (127.90 ± 14.81) on day 1. No differences were found in hematocrit and erythrocytes.

Overall, hematocrit, hemoglobin, and erythrocyte values were higher in CT + I than in CT + V. Along the clinical follow-up, the mean value of hematocrit (L/L), hemoglobin (g/L), and total erythrocytes (1 x 10^{12}/L) in CT + I group was 0.34 ± 0.06, 113.90 ± 25.40 and 5.43 ± 1.16, respectively; while in CT + V group was 0.31 ± 0.06, 102.80 ± 18.70, and 5.07 ± 0.93.

The concentration of plasma proteins showed no significant differences between the evaluated treatments (data not plotted). On the first three days, its values were similar between groups (CT + I = 45.73 ± 9.13 g/L *vs* CT + V = 44.36 ± 10.33 g/L). However, on days 4 and 5, considered recovery days, CT + I showed higher concentrations (day 4 = 44.67 ± 11.43 g/L; day 5 = 47.89 ± 14.05 g/L) than CT + V (day 4 = 35.33 ± 2.52 g/L; day 5 = 36.33 ± 4.62 g/L).

No statistical differences in neutrophil counts were identified between groups at the beginning of the study. The group with CT + I exhibited a significantly lower value than those observed on days 2 and 4 in CT + V (Fig. 3A and B). In the analyses per group, we observed that on days 0, 2, and 4, the group with CT + V showed significant differences when comparing day 4 *vs* day 0. Although there were no statistical differences, it must be considered that the neutrophil count was lower on day 2 *vs* day 4 (Fig. 3C). In the critical period (day 2), we observed a decrease in the number of neutrophils initially reported on day 0 and that detected on day 4, which resulted in the differences observed in CT + I, as shown in Fig. 3D.

At the beginning of the clinical follow-up, no differences were detected in the range of the number of lymphocytes between groups. Lymphocytes were compared between treatments on the critical day (day 2) and showed significant differences ($P \leq 0.045$) (Fig. 4A); however, no differences were found on the recovery day (day 4). On day 2, CT + I exhibited a lower count than CT + V (Fig. 4A and B). When comparing days 0, 2, and 4 per treatment, the CT + I group presented a significant increase in lymphocytes in the recovery period *vs* days 0 and 2 (Fig. 4D). Although CT + V showed no significant differences (Fig. 4C), there was an increase in lymphocytes on day 4.

Hormone parameters

Cortisol is the most often used endocrine biomarker in CPE management. Concentrations ≥ 224 nmol/L at 24 and 48 h postadmission have been linked to a higher mortality [21]. Only one of the 6 dead patients showed values above 224 nmol/L at 24 h of hospitalization.

Plasmatic concentrations of cortisol in both groups were equivalent (data not plotted). When comparing treatments, there were differences ($H = 21.17$, $d = 60,1$; $P \leq 0.012$) since CT + I showed higher values (97.43 ± 93.97 mmol/L) than CT + V (69.77 ± 58.99 mmol/L). Cortisol concentrations (mmol/L) in CT + I on day 1 were 149.9 ± 96.13 and of 151.30 ± 110.90 on day 2. Those of CT + V were 127.90 ± 51.86 on day 1 and 89.65 ± 65.30 on day 2. Evidently, the group with CT + I showed higher cortisol values than the group with CT + V on the days considered as critical period; in both groups there was a decrease from day

1 with respect to basal values. A similar reduction in cortisol levels due to immunomodulator consumption has been reported in human patients [22].

Neurochemical parameters

Plasmatic concentrations of neurotransmitters were evaluated on days 0, 1, and 2, considered the critical period of the disease. The comparison of plasmatic NE levels between treatments showed significant difference on day 1 (Fig. 5A). It is clearly seen that norepinephrine concentrations are higher in the CT + I group than in CT + V in the times evaluated. No differences were observed in plasmatic EP (Fig. 5B). The CT + I group showed EP values lower on day 1 and on day 2 than the baseline value. The SE values presented in Fig. 5C evidence an increase only in CT + I on day 2 of follow-up ($H = 12.93$, $d = 60,1$; $P \leq 0.0240$). These high NE concentrations in the CT + I group suggest the neuroimmunomodulation mechanisms are active secondary to the consumption of the immunomodulator.

In silico analysis of dog CXCR4 interacting with human mUb and UbΔGG

Given that human mUb and UbΔGG are the major components in the immunomodulator used as therapeutic auxiliary in this study, and due to Ub has been reported as an agonist of human CXCR4 [18], we performed an *in silico* evaluation of how dog CXCR4 interacts with mUb and UbΔGG (Fig. 6A and B) to test the feasibility of canine receptor could recognize these two molecules. We obtained values of constant dissociation (Fig. 6C); although the values are not identical, there are no significant statistical differences between the values identified for the corresponding Kd. From a biological standpoint, this means that the major components of the immunomodulator can indistinctively bind to dog CXCR4 while the binding affinity is not affected by the loss of both glycines.

Discussion

CPE is the most common and severe viral gastrointestinal disease among puppies aged 6 months [3]. The development of sepsis during CPE is frequent and linked to bacterial translocation and lymphopenia, physiopathological alterations of the disease [3, 4].

Sepsis is a potentially deathly organic dysfunction caused by a deregulate inflammatory response in the host against an infectious stimulus [23]. In sepsis, the systemic activation of the innate immune response through pattern recognition receptors (PRRs) [24] leads to the production and release of proinflammatory mediators, such as IL-1β, TNF-α, IL-6, IL-12, CRP, macrophage migration inhibitor factor (MIF), and high mobility group box 1 protein (HMGB-1), particularly by macrophages [25, 26]. The first contact of immune system with a pathogen should be enough to trigger a well-regulated inflammatory response able to coordinate the elimination of the antigen source and self-limit the immune response. Still, inflammatory response amplification systems, as the activation of the complement and coagulation systems, increase the severity of the inflammatory process and perpetuate it. They promote tissue damage that causes the release of damage-associated molecular patterns (DAMPs), which in turn induce a second inflammatory

wave and limit adequate regulation [27]. It has been reported that puppies with CPE show high concentrations of TNF-α, IL-1β, and CRP [28, 29] which reflect a hyperinflammatory status.

In this study, we have demonstrated that the administration of an immunomodulator as an auxiliary treatment for CPE patients increases survival. Our CT + I group showed an increase of ≈ 40%, higher than that obtained with a conventional treatment. It has been described that the immunomodulator used as an auxiliary treatment in this study can successfully modulate the inflammatory response in pediatric patients with sepsis. It increases the patients' survival by reducing CPR concentration and the number of circulating neutrophils [14].

The vagal cholinergic pathway (VCP) is mediated by efferent vagal fibers that synapse with neurons of vagus nerve branches widely distributed in the organism, which then release acetylcholine (ACh) at the synaptic cleft. It can bind to the α7-nicotinic receptor (α7-nAChR) of macrophages to transcriptionally inhibit the production of proinflammatory cytokines as TNF-α, IL-1β, and IL-6 [19].

In the splenic sympathetic pathway (SSP), vagal nervous (VN) stimulates the splenic sympathetic nerve, releasing NE. Through the β2-adrenergic receptor, NE promotes ACh production by an overpopulation of splenic CD4 + T lymphocytes. The lymphocytes express acetylcholine transferase (AChT), the enzyme that catalyzes ACh production from choline and acetyl-CoA [30].

The importance of the vagal modulation of the inflammatory response has been evidenced in multiple *in vivo* animal models [31]. In them, vagal stimulation increases survival by significantly reducing proinflammatory cytokine levels [32, 33].

In a murine model of infection with Herpes virus type 1, the administration of the immunomodulator used in this work reduced the serum concentrations of TNF-α and IL-6, leading to an increase in survival [34]. These data suggest that the immunomodulator can reduce the circulatory levels of inflammatory cytokines, activating VN by binding mUb and UbΔGG to CXCR4.

This study did not quantify cytokine concentrations; however, the cholinergic activity induced by the immunomodulator can be inferred by the decrease in cortisol and EP concentrations, which were assessed in our study groups. The hypothalamic–hypophyseal–adrenal (HHA) axis is activated by humoral pathways, releasing cortisol. The humoral pathway activates the HHA axis by registering high concentrations of proinflammatory cytokines and other inflammatory mediators [35]. The acute cortisol release hampers the inflammatory response [36]; still, a hyperactive HHA leads to increased and constant cortisol levels that can generate immunosuppression and raise mortality [37].

The reduction in plasma cortisol observed since day 1 in the surviving CT + I patients suggests that the immunomodulator increases the vagal tone in patients while reducing the proinflammatory environment and thus cortisol levels are lower. This is supported by the measurements of EP in the CT + I group during the critical period (days 1 and 2). The levels were like those found in the surviving CT + V patients. This suggests that the parasympathetic pathway of the VN inflammatory modulation is active. Other studies

have described an inverse relationship between the parasympathetic vagal tone and EP concentration (sympathetic modulation) [38].

The reduction in circulating neutrophils and lymphocytes observed in CT + I on day 2 of hospitalization also evidences the regulation of the inflammatory process. Several *in vitro* studies have proven that when bound to their ligand, β2-adrenergic receptors negatively modulate the neutrophil oxidative burst, chemotaxis, the formation of neutrophil extracellular traps (NETs), and the expression of adhesion molecules, leukotriene B4 (LTB4), chemokines, and cytokines [32, 39]. The effects of ACh on neutrophils have been investigated using nicotine as an α7-nAChR agonist. For instance, nicotine increases survival in endotoxemia and experimental sepsis when inhibiting the production and release of systemic inflammatory mediators as TNF-α and HMGB-1 [32, 40], and reduces oxidative multiorgan damage induced by neutrophils [41].

Additionally, it has been demonstrated that the activation of nicotinic receptors can inhibit the massive recruitment of neutrophils in vital organs, preserving tissue integrity [39]. This could explain the reduction in neutrophils in CT + I on days 2 and 4 *vs* CT + V despite other factors of the disease to reduce circulating neutrophils, as a reduction in hematopoietic production [42]. This same finding has been reported in pediatric patients with sepsis [14].

Although ubiquitin activates VN, it can also directly bind to CXCR4 expressed in immune system cells to reduce the proinflammatory activity [16, 17]. Still, CPE has an acute course that demands a fast and efficient modulation of the inflammatory response, such as neuroimmunoregulation mechanisms [43], unlike cell and humoral response at their peak activity. Therefore, we postulate that neuroimmunoregulation could play a key role in the increase of survival of CPE patients.

From the first clinicopathological descriptions of the disease, the quantification of blood cells has been used as a severity and prognosis biomarker due to their low cost and accessibility [44]. Hematological alterations are generated through several physiopathological mechanisms, such as the viral destruction of hematopoietic precursors in the bone marrow by CPV-2 tropism. This leads to a reduction in the number of circulating neutrophils, lymphocytes, erythrocytes, and platelets [21].

The circulating neutrophil and lymphocyte counts are the most common prognosis biomarkers of CPE. The concentration of circulating neutrophils ($\geq 3.0 \times 10^9$/L) has a positive predictive value of 95% at the moment of admission [45]. Fifty-five percent of the patients in the study (10/18) showed numbers below the predictive value and half of them died: 4 in the CT + V group and 1 patient in CT + I. In addition, the counts lymphocytes less than 1.0×10^9/ L at the time of hospitalization has a predictive value for mortality of 98%. In the CT + I group, all patients who died exhibited lymphocyte counts below this value; but two patients with this condition survived. In the CT + V group, only two of the four patients who died showed cell counts below 1.0×10^9/L [46]. This suggests that neutrophil and lymphocyte counts as prognosis biomarkers are not entirely accurate.

A key hematological finding in CPE is anemia. Its presence can be explained by the decrease in erythrocyte production. Other factors can affect the number of circulating erythrocytes, such as blood loss secondary to hemorrhagic gastroenteritis, caused in turn by the destruction of intestinal crypts and the rupture of the intestinal barrier [21]. Even though the immunomodulator has been reported to have hematopoietic effects [47], there are no reports on whether they cover the red line blood cells.

In addition, CPE patients show a decrease in plasma proteins, mainly due to hypoalbuminemia and hypogammaglobulinemia [48]. The latter is caused by the loss of gastrointestinal blood, enteropathy with protein loss secondary to the destruction of intestinal crypts, and the increase in vascular permeability associated to SIRS. Hypogammaglobulinemia is the result of B-lymphocyte depletion secondary to thymic atrophy [21]. The CT + I group showed higher plasma protein concentrations on days 4 and 5 (recovery period) as compared against CT + V. If we link these findings with other variables, CT + V likely suffered a greater damage in the intestinal epithelium, leading to an extended enteric protein loss and the consequent decrease on the recovery days. This is supported by the high scores in the clinical evaluation, the decrease in red line cells, and the weight loss, variables that presented a better behavior in the CT + I group.

There is no sex disposition to develop CPE among dogs younger than 6 months, but it has been documented that intact males are more likely to develop the disease than females [3]. Although all breeds are susceptible to CPE, Rottweilers, Doberman Pinschers, American Pit Bull Terriers, Labrador Retrievers, English Springer Spaniels, and German Shepherds are at a higher risk [1, 13]. It has also been documented that mixed-breed dogs are less susceptible to CPE than their pure breed counterparts [3] while other researchers have found no breed predisposition [42]. The lack of vaccination is the main risk factor to contract CPE [48]. The vaccinated dogs are at a lower risk of developing CPE than those unvaccinated [44]. Decaro et al. [5] state that the development of CPE in immunized dogs is due to five causes: the persistence of maternal immunity at the time of vaccination, vaccination in non-responsive patients, the presence of different antigenic variants, an inadequate vaccine storage, and deficient vaccination protocols.

Study limitations: To adequately benefit from the results obtained in this work, we must state that among the limitations is the number of recruited animals, mostly CT + V. Due to the high mortality rate of the disease, the sample was considerably reduced. Future studies should consider a larger control group so that the number of surviving participants allow for more efficient statistical comparisons. In addition, further works should consider strategies to measure soluble inflammatory parameters as cytokines, among others. To the best of our knowledge, this is the first work to report the interspecies effect of this human immunomodulator in dogs as well as the first report through in silico analysis showing the potential molecular interaction between mUb or UbΔGG with dog CXCR4. As suggested by the results of *in silico* molecular coupling, the positive effects observed in this work are likely due to human mUb/UbΔGG and dog CXCR4, triggering vagal modulation mechanisms previously described.

Conclusions

The immunomodulator employed in this work increases the survival of patients with CPE by modulating the number of neutrophils and lymphocytes in the critical stage of the disease, probably associated with the neuronal modulation (due to the concentrations of cortisol and epinephrine). This is shown in improved clinical signs and associated parameters. These encouraging findings indicate the efficacy of the immunomodulator as a therapeutic aid and could consolidate its use as a treatment option in CPE.

Further studies that should be done to consolidate the findings of this work.

Materials And Methods

Study population

Eighteen patients with CPE from five veterinary hospitals in the metropolitan area of the Valley of Mexico were included between March and September 2019. All the puppies met the inclusion criteria of the research protocol (FTU/P3/19/03) as approved by the Ethics in Research Committee of Escuela Nacional de Ciencias Biológicas at Instituto Politécnico Nacional (ENCB-IPN), equivalent to IACUC regarding Handling and Care of Animals. All the tutors signed informed consent letters. The manuscript follows the recommendations in the ARRIVE guidelines and all experiments were performed in accordance with relevant guidelines and regulations.

The inclusion criteria of this study can be consulted in Table S1 of supplementary information. We included CPE patients under 6 months of age, regardless of sex and breed, that exhibited at least two clinical criteria of SIRS secondary to sepsis (hypothermia < 37.8 ºC, fever > 39.4 ºC, tachycardia > 140 bpm, tachypnea > 30 breaths/min, leukopenia < 5.5 x 10^9/L, and leukocytosis > 12.5 x 10^9/L) [7]. Patients were randomly assigned to any of the two study groups. The group with conventional treatment plus vehicle (CT+V, n = 7) or the conventional treatment plus immunomodulator group (CT+I, n = 11). Once the patients were approved for the study, they were admitted and remained under the care of veterinary doctors at the participant center.

Immunomodulator

The immunomodulator used is a hDLE, commercialized as Transferon (batch 18A03), produced for this protocol by Pharma-FT at ENCB-IPN (Mexico City, Mexico) [49]. Briefly, the leukocyte-platelet concentrates of healthy human donors were collected at blood banks in Mexico City and lysed through freezing/thawing cycles. After lysis, the active pharmaceutical ingredient (API) was obtained with tangential flow filtration, using a 10-kDa molecular weight cut-off cassette. It was especially formulated for this study with 0.5 mg/mL total protein concentration using UV absorbance at 280 nm. The final product passed the identity test by molecular size chromatography, endotoxin content by Limulus amebocyte lysate, and microbial limits according to the Pharmacopeia of the United Mexican States [50]. Then, the hDLE was stored in 10 mL sterile glass bottles with bromobutyl stoppers in laminar flow cabinets and handed over to the head researcher of the study along with the same number of vials

containing water for injection (Laboratorios PiSa, Jalisco, Mexico). Both products were stored and transported in refrigeration (5 ± 3 ºC) to the administration site.

Administration of conventional treatment and immunomodulator

Patients were randomly assigned to two different groups once tutors gave their consent. Both groups were handled conventionally as recorded in the files of each patient. In addition, the control group (CT+V) received the vehicle, water for injection, and the other group (CT+I) was administered the immunomodulator (0.5 mg in 1 mL vehicle, daily). Treatments were administered subcutaneously in the interscapular region every 24 h for 5 days.

Sample collection: feces and whole blood

In all the cases, two fecal samples were collected using a sterile swab directly in the rectum. The first one was used as screening using rapid detection of CPV-2 by immunochromatography (Anigen Rapid CPV Ag Test kit, Bionote, South Korea) and the second helped confirm the diagnosis by endpoint polymerase chain reaction (PCR). The sample was placed in a test tube without anticoagulant (BD Vacutainer®, New Jersey, USA) with 1 mL sterile saline solution and kept at –20 ºC. It was then placed in a sterile microcentrifuge tube and centrifuged at 12,600 x *g* for 10 min. We added DNAzol (Invitrogen®, USA) to the supernatant obtained (400–600 µL) at a 1:1 ratio and kept it at –75 ºC until the extraction of genetic material.

Blood samples were extracted from the jugular vein at days 0, 1, 2, 3, 4, and 5. The blood was placed in tubes with anticoagulant EDTA (BD Vacutainer®, New Jersey, USA) and kept under refrigeration (5 ± 3 ºC) until its analysis. The amount obtained (1–3 mL) was adjusted to the patient's weight per day, caring for the well-being of the animal. Once the hematological parameters were assessed, samples were centrifuged at 1,233 x *g* for 10 min. The plasma obtained was stored at –20 ºC until cortisol and neurotransmitters were quantified. The samples were obtained between 9 a.m and 12 p.m.

Confirmation of infection by CPV-2

Infection was confirmed at the Virology Laboratory of the Facultad de Medicina Veterinaria y Zootecnia (FMVZ) at Universidad Nacional Autónoma de México (UNAM). To extract the genetic material, 500 µL DNAzol (Invitrogen®, Massachusetts, USA) and 8 µL Proteinase K (Bioline, London, UK) were added at a concentration of 60 µg/mL to the stored sample (500 µL) at –75 ºC. The mixture was incubated at 56 ºC for 30 min and DNA was extracted according to the DNAzol reagent protocol, using 100 and 75% molecular biology grade ethanol (Sigma Aldrich, Darmstadt, Germany). The precipitate was hydrated with 30 µL DNAse-free water, and DNA was quantified by spectrophotometry in a NanoDrop spectrophotometer. The endpoint PCR was carried out following the protocol of Master Mix Platinum II PCR MM (Invitrogen®, Massachusetts, USA) in a MultiGene® Optimax thermal cycler with 30 denaturation cycles at 95 ºC for 30 s, annealing at 55 ºC for 30 s, and extension at 72 ºC for 30 s. The primers used, forward 5'-GAC CAG CTG AGG TTG GTT ATA G- 3' and reverse 5'- GGT GCA TTT ACA TGA

AGT CTT GG 3', were directed to the gene coding for the capsid protein (VP2) of CPV-2 and created a 466-bp amplicon. They were designed by the research group at FMVZ, based on the publication by Balasubramaniam et al [51]. The electrophoresis was carried out in a 2% agarose gel stained with ethidium bromide for analysis in a UV transilluminator.

Clinimetric evaluation, weight, survival time, and days of hospitalization

The clinical evaluation took place across 5 days, between 8:00 a.m. and 11:00 a.m., according to the guidelines of the diagnostic methodology to perform a detailed clinical exam. The veterinarians in charge of the patients received training before the study to homologate clinical evaluation criteria. The parameters evaluated were temperature, mental health (alert and responsive, depressed or in coma), dehydration percentage, degree of abdominal pain, characteristics of feces (pasty stool, mucoid diarrhea, hemorrhagic diarrhea), presence and characteristics of vomit, and death [7]. Each parameter was given a measuring scale; a higher score indicated the severity of the disease was greater. Table S2 of supplementary information specifies the clinical parameters evaluated as well as their scores. Weight was recorded during the clinical inspection.

Survival time record started 24 h after patients were admitted and ended at day 5. Days of hospitalization were quantified up to hospital discharge, which took place once the patients consumed feed and water in the absence of vomit for more than 24 h and showed no clinical signs of depression, fever, or hypothermia.

Hematological parameter assessment

Blood samples were sent to a private veterinary clinical laboratory for a complete hemogram. Hematocrit, hemoglobin, and total erythrocyte and leukocyte counts were assessed using a CELL-DYN® Emerald hematology analyzer (Abbot, Illinois, USA), which is based on electronic impedance and absorption spectrophotometry. The analyzer was calibrated and verified for the target species. The number of circulating neutrophils and lymphocytes was obtained from a blood smear with a differential leukocyte count. This information was used to obtain the absolute value based on the total number of leukocytes. Plasma proteins were identified in a conventional refractometer (RHC-200act, Mexico).

Plasmatic cortisol quantification

Plasmatic cortisol was determined in a private veterinary clinical laboratory. The plasma samples were processed in an Architect i2000SR immunoassay analyzer (Abbot, Illinois, USA). The method was based on a heterogeneous chemiluminescent microparticle immunoassay.

Quantification of plasmatic norepinephrine, epinephrine, and serotonin

Norepinephrine (NE), epinephrine (EP), and serotonin (SE) were determined at the Psychoimmunology Laboratory, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz. We extracted NE, EP, and SE from 250 µl plasma by adding 250 µl extraction buffer containing 5% ascorbic acid, 200 mM sodium

phosphate, 2.5 mM L-cysteine, 2.5 mM EDTA, and 2.4 M perchloric acid. The mixture was incubated at –20 ºC for 10 min. The supernatants containing NE, EP, and SE were collected after centrifugation at 12,419 x *g* and 4 ºC for 10 min. They were processed by solid-phase extraction (SPE) using a Hypersep C18 cartridge (Thermo Scientific, Massachusetts, USA) without activation to retain lipids and recover neurotransmitters, and samples were passed through 0.22 µm filters. Neurotransmitter concentrations were identified by reversed-phase HPLC (RP-HPLC) in a system consisting of a PU-2089 plus pump (Jasco Inc., Japan) and an X-LC™ 3120FP fluorescence detector (Jasco Inc., Japan). The instruments were controlled using ChromNav software (Jasco Inc., Japan). Chromatographic runs were carried out with a Jupiter C18 column (300 Å, 5 µ, 4.6 x 250 mm, Phanomenex®) at 30 ºC. The column was in equilibrium with mobile phase A containing 0,1% trifluoroacetic acid in water. A linear gradient was run from min 5 to min 20 with mobile phase B containing 0,1% trifluoroacetic acid in acetonitrile. The flow rate was 0.8 mL/min. The fluorescence detector was set at a gain of 1000, attenuation of 32, response of 20 s, excitation at 280 nm and emission of 315 nm. The injection volume of the sample was 80–100 µL.

In silico analysis of the interaction between dog CXCR4 and human mUb and UbΔGG

The crystallized structure of human CXCR4 contained in the Protein Data Bank (PDB; www.rcsb.org) ID. 4RWS was edited using Biovia Discovery Visualizer Studio software (BDVS, Discovery Studio Modeling Environment, 2017; Dassault Systèmes) to eliminate accessory proteins (vMIP2 and T4 lysozyme). In addition, the intramolecular loops of human CXCR4 were recovered using the *Protein Preparation Wizard* tool of Maestro v12.5 (Schrödinger Suite, Protein Preparation Wizard, 2021-1; Schrödinger). Edited human CXCR4 was used to model dog CXCR4 (*Canis lupus familiaris*; NP_00101041491.1) by homology in Modeller v9.25 [52]. The model with the lowest DOPE (Discrete Optimized Protein Energy) score was chosen among the five candidate models.

To carry out the homology modelling of mUb and UbΔGG, the crystallized structure of 6KDU obtained from PDB was used as a model after edition with BDVS to eliminate accessory molecules (adenine 5-phosphoribose, Zn + 2, and Mg + 2). The model with the lowest DOPE score was chosen among the candidates for mUb and UbΔGG.

To couple dog CXCR4 to human mUb or UbΔGG, we used a masking template generated on PyMOL v2.0 (The PyMOL Molecular Graphics System version 2.0; Schrödinger) to direct the interaction specifically in the extracellular domain of CXCR4. The coupling was generated using the ClusPro 2.0 server [53], choosing the interaction mode *Electrostatic-favored*. The 10 major groups obtained were analyzed to identify the dissociation constant at 37 ºC with the PRODIGY server (prediction PROtein binding enerGY). The coupling visualization was produced with PyMOL software.

Statistical analysis

All statistical tests were run on GraphPad Prism v8.0.1 (San Diego, California, USA). In all cases, data were classified by group or evaluated time and were applied a Shapiro-Wilk test to assess normality. Once two groups with normal distribution were compared, one-way ANOVA and Tukey's post-hoc test were

applied. Alternatively, Kruskal-Wallis and Dunn's post-hoc tests were used. To compare the age of patients and period of hospitalization, Student t and Mann-Whitney tests were applied, respectively.

When comparing weight in all the cases, we used the values resulting from the weight change percentage formula (see Equation 1)

$$Weight\ change\ percentaje = \left[\left(\frac{final\ weight - initial\ weight}{initial\ weight}\right) * 100\right] \quad (1)$$

Then, Kruskal-Wallis and Dunn's post-hoc tests were carried out. The data analysis of the clinical score was using a Kruskal-Wallis test was done. The survival was assessed with a Kaplan-Meier survival curve and a Gehan-Breslow-Wilcoxon post-hoc test. To evaluate the differences induced by the treatments evaluated in circulating neutrophil and lymphocyte counts, we used the values obtained on days 0, 2, and 4. The neurotransmitter comparison was evaluated on days 0, 1, and 2 through a Kruskal-Wallis and Dunn's post-hoc tests. In all cases, there was a statistical difference when $P \leq 0.05$, and data were described with mean ± SD.

Abbreviations

CPE: canine parvoviral enteritis; SIRS: systemic inflammatory response syndrome; mUb: monomeric ubiquitin; UbΔGG: ubiquitin without the last two glycine residues; CXCR4: - C-X-C motif chemokine receptor 4; CT: conventional treatment; V: vehicle; I: immunomodulator; CPV-2: Canine parvovirus type 2; MODS: multiple organ dysfunction syndrome; hDLE: human dialyzable leukocyte extract; CRP: C-reactive protein; NE: norepinephrine; EP: epinephrine; SE: serotonin; VP2: capside protein 2; PRRs: pattern recognition receptors; IL-1β: interleukin 1-β; TNF-α: tumoral necrosis factor α; IL-6: interleukin 6; IL-12: interleukin 12; MIF: macrophage migration inhibitor factor; HMGB-1: high mobility group box 1 protein; DAMPs: damage-associated molecular patterns; VCP: vagal cholinergic pathway; ACh: acetylcholine; α7-nAChR: α7-nicotin receptor; SSP: splenic sympathetic pathway; VN: vagal nervous; AChT: acetylcholine transferase; HHA: hypothalamic-hypophyseal-adrenal; NETS: neutrophil extracellular traps; LTB4: leukotriene B4.

Declarations

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Author contributions

AI M, LC-M, and SM P-T generated the research idea and developed the proposal. AI M, L V-C, AL F, and L P carried out the statistical analysis, analyzed data, edited, and organized the manuscript. S V-L was in charge of production and packaging logistics of the immunomodulator. G P-S quantified the neurotransmitters. R S-C analyzed the molecular coupling. G M-S reviewed and edited the original draft. All the authors have read and approved the manuscript for its publication.

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Availability of data and materials

All the data sets generated and/or analyzed along the study that support our findings are not publicly available since this work is part of the research at UDIBI, ENCB-IPN. However, they are available through the corresponding author at a reasonable request at any time.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of ENCB-IPN (FTU/P3/19/03). All tutors of the recruited patients gave their written consent.

Consent for publication

Non-applicable.

Competing interests

SM P-T is the manager of UDIBI, ENCB-IPN, responsible for the commercialization of Transferon, she participated in the development of the concept and not in the data analysis. The rest of the researchers are dedicated to the investigation of this immunomodulator and do not have competitive economic interests, for instance, financial, employment, or personal economic interests. They also do not have competitive non-financial interests, such as unpaid membership in a government or non-governmental organization, unpaid membership in an advocacy or lobbying organization, unpaid advisor position in a business organization, writing or consulting for an educational company, acting as an expert witness or any other.

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Figures

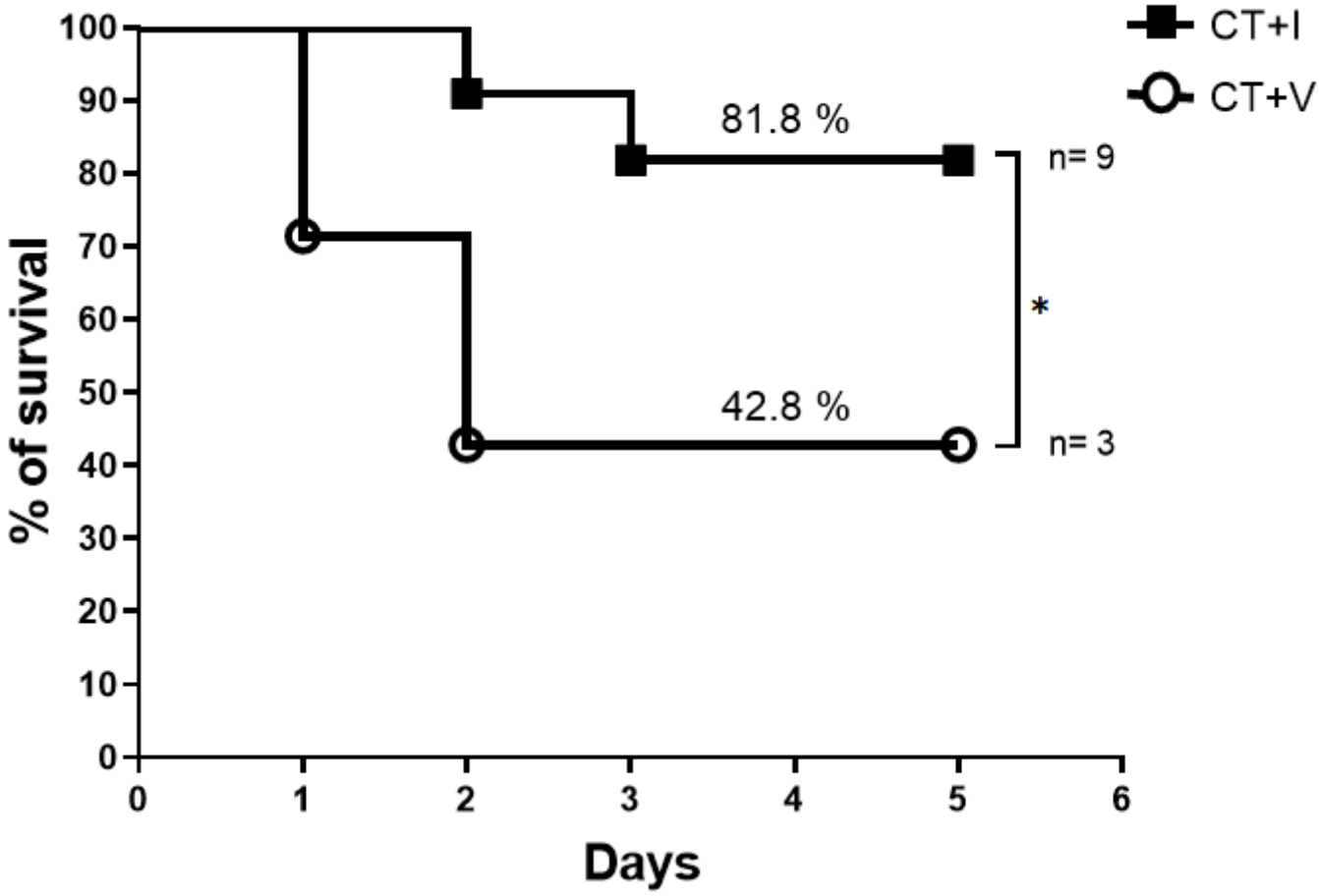

Figure 1

Effect of an immunomodulator over survival of puppies affected by CPE. Patients administered 0.5 mg of immunomodulator daily sowed an increase in survival (%) vs CT+V group. Initial number of patients in CT+V group was 7 and final number was 3. Initial number of patients in CT+I group was 11 and only 2 died. The difference in survival percentage is 39.0%. Kaplan-Meier test, Gehan-Breslow-Wilcoxon post-hoc test. *P ≤ 0.05. CT= conventional treatment; I= immunomodulator; V= vehicle.

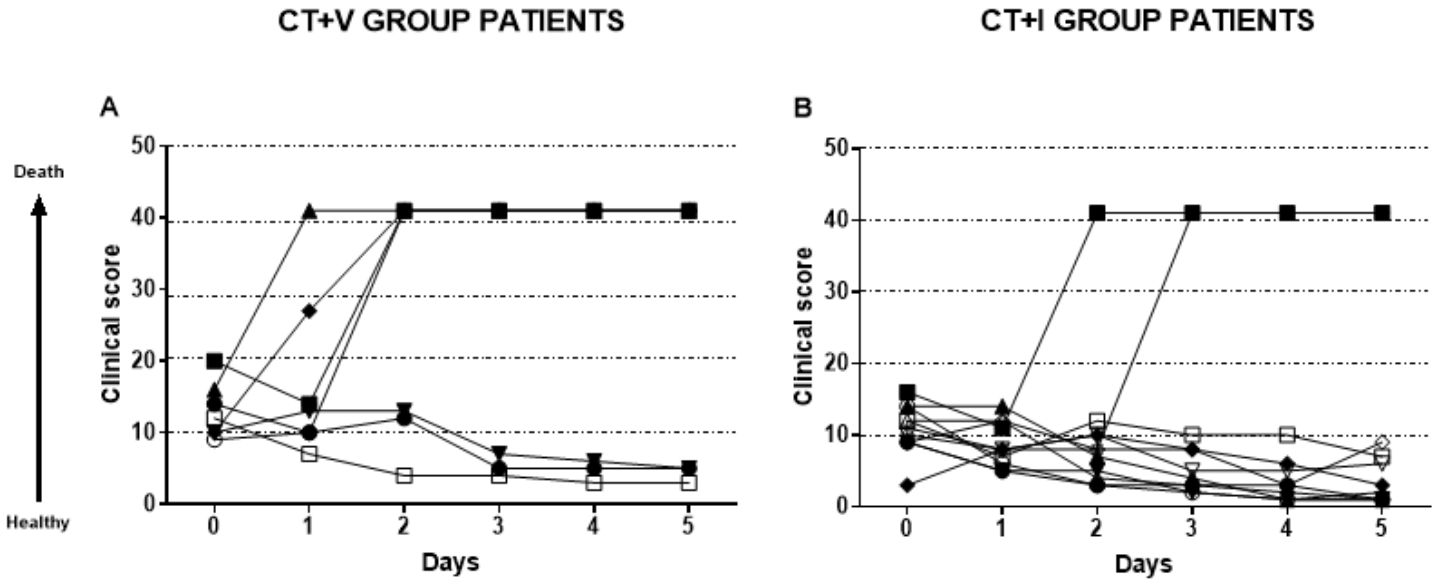

Figure 2

Effect of an immunomodulator over clinical scores of puppies affected by CPE. (A) Clinical score of CT+V across time. On days 1 and 2 surviving patients of CT+V showed higher values than survivors of CT+I (B). A decrease in clinical scores in both groups is shown on day 3. Each symbol represents a patient. Score of dead patients is 41. Kruskal-Wallis test, H= 19.99, d = 90,1; P≤ 0.018. *P ≤ 0.05. CT= conventional treatment; I= immunomodulator; V= vehicle.

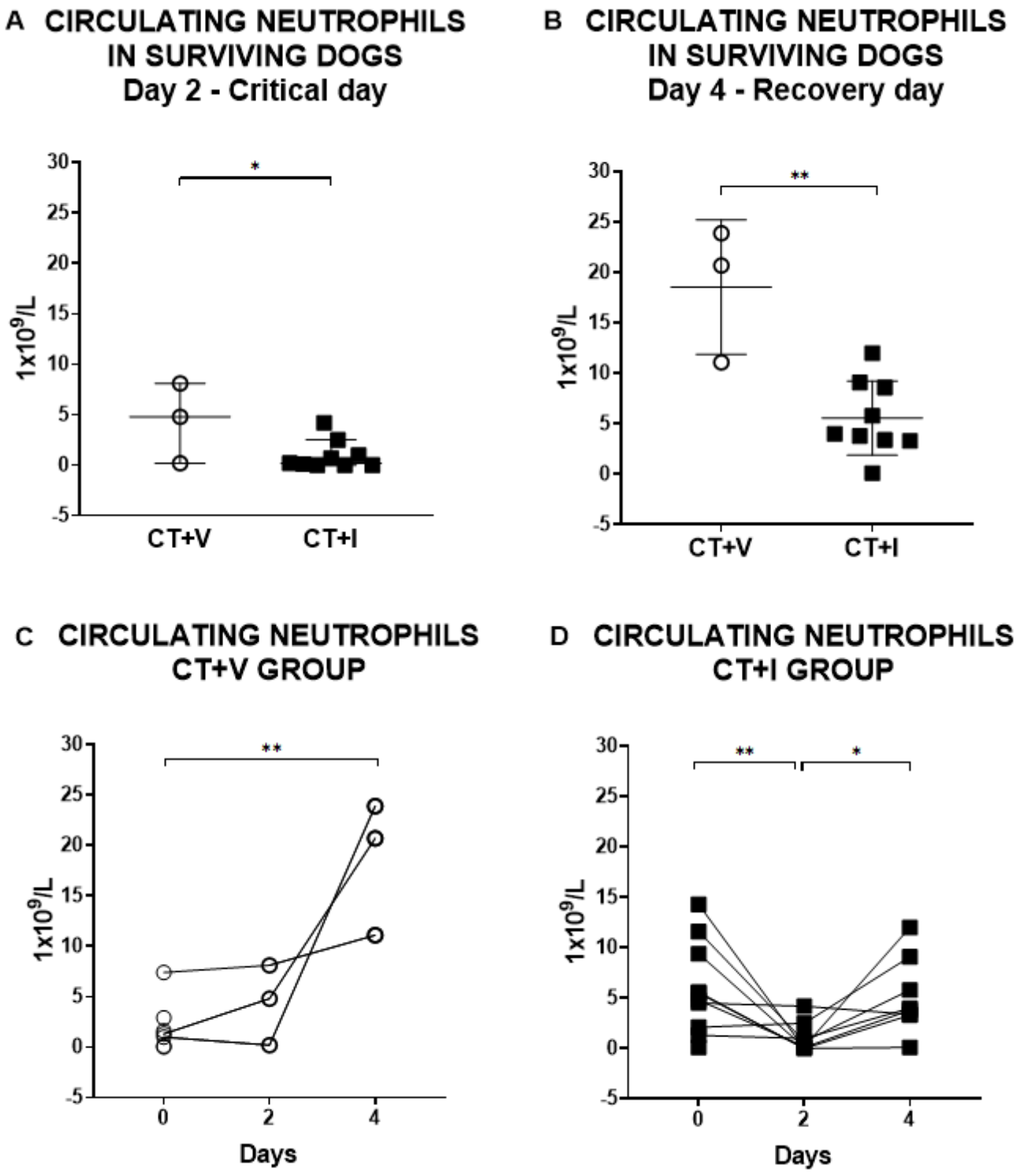

Figure 3

Effect of an immunomodulator over circulating neutrophil concentration of puppies affected by CPE. (A) Circulating neutrophils in surviving dogs (day 2- critical day) and (B) circulating neutrophils in surviving dogs (day 4-recovery day), statistical differences on days 2 and 4 are due to lower neutrophil concentrations in CT+I than in CT+V. Mann-Whitney test. Each dot represents a patient. Median ± 5–95% confidence interval. (C) Neutrophil concentrations on day 0, critical day (2) and recovery day (4) in CT+V

group. Neutrophil concentrations on day 4 are responsible for statistical differences. (D) Circulating neutrophils on day 0, critical day (2) and recovery day (4), reduction in neutrophils on day 2 results in statistical differences in CT+I. Each dot represents a patient. Kruskal-Wallis test, Dunn's post-hoc test. *P ≤ 0.05, **P ≤ 0.01. CT= conventional treatment; I= immunomodulator; V= vehicle.

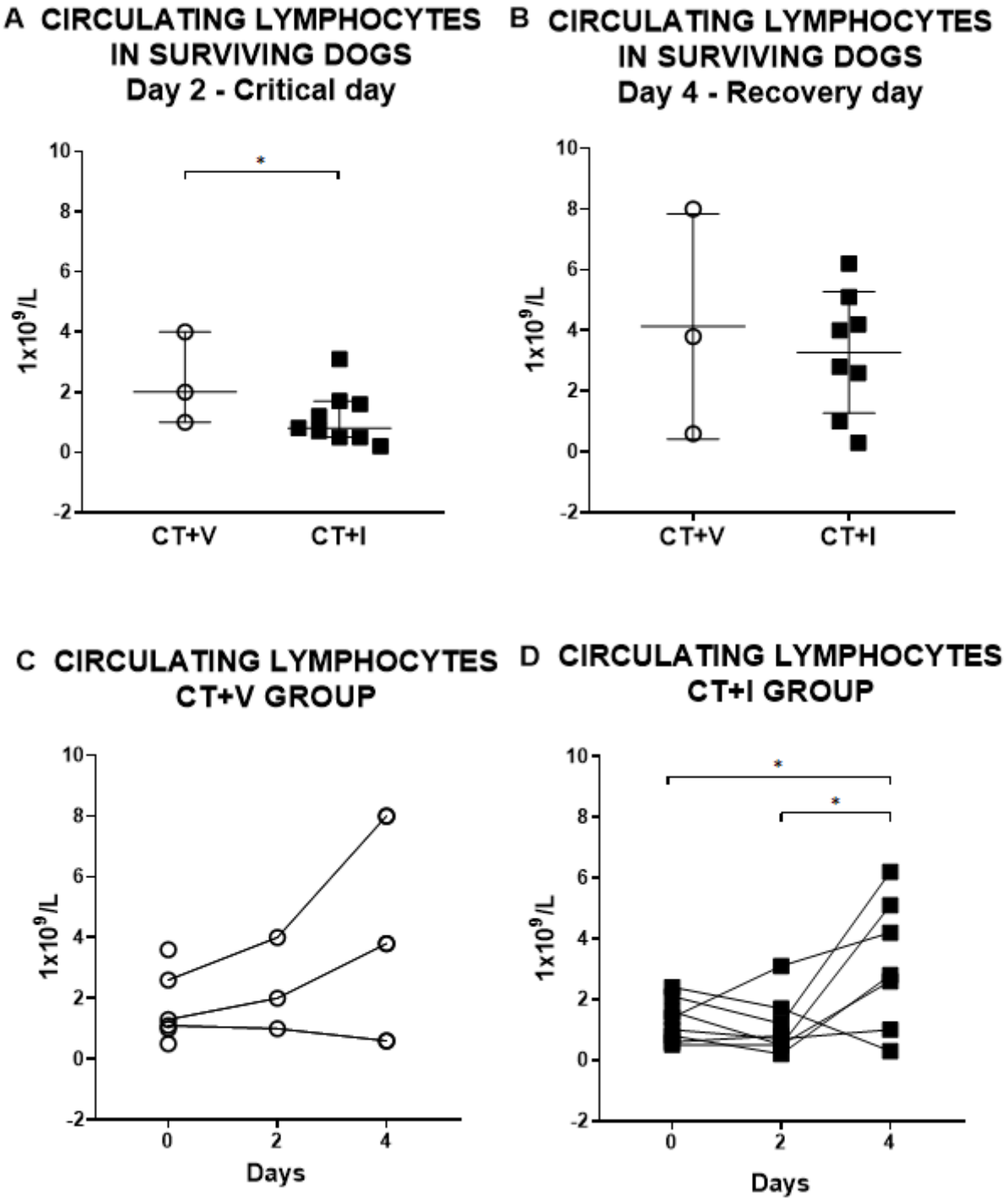

Figure 4

Effect of an immunomodulator over the concentration of circulating lymphocytes of puppies affected by CPE. (A) Circulating lymphocytes in surviving dogs on critical day (day 2) and (B) circulating lymphocytes

in surviving dogs on recovery day (day 4); there are no statistical differences between groups on day 4; the difference on day 2 is due to the lower lymphocyte values in CT+I vs CT+V. Mann-Whitney test. Each dot represents a patient. Median ± 5–95% confidence interval. (C) Neutrophil concentrations on day 0, critical day (2), and recovery day (4) in CT+V group. Lymphocyte concentrations do not show statistical differences at any time evaluated. Kruskal-Wallis test, Dunn's post-hoc test. Each dot represents a patient. (D) Circulating lymphocytes in CT+I group, this group showed differences in recovery time due to an increase in lymphocytes as compared with basal measurement and day 2. One-way ANOVA, Tukey's post-hoc test. Each dot represents a patient. Mean ± SD. *P ≤ 0.05. CT= conventional treatment; I= immunomodulator; V= vehicle.

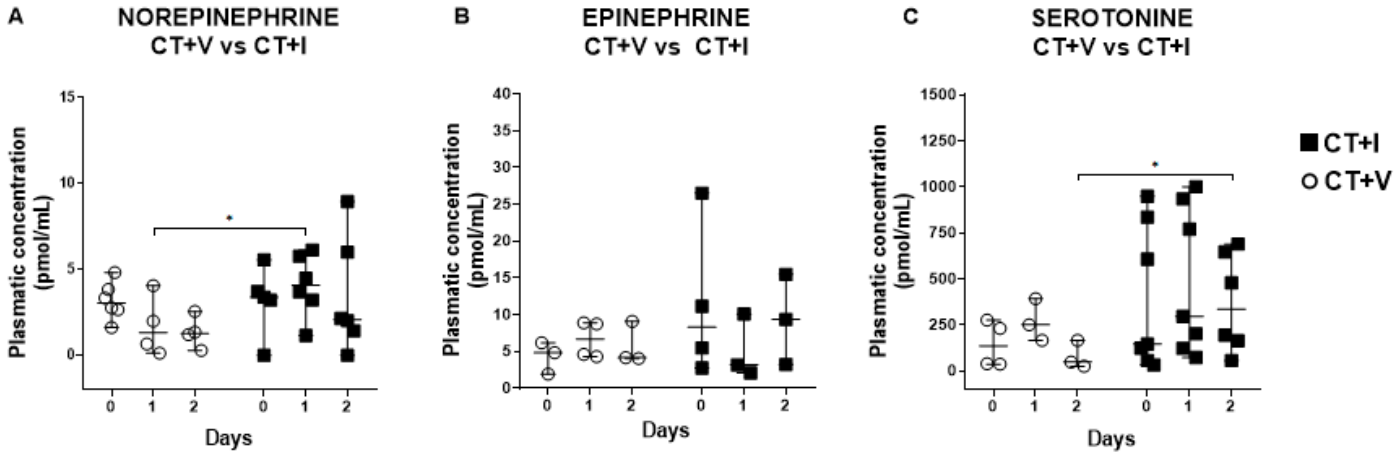

Figure 5

Effect of an immunomodulator over the plasma neurotransmitter concentration of puppies affected by CPE. (A) Norepinephrine: A significant increase was observed in the CT+I group on day 1 of evaluation with respect to CT+V group. (B) Epinephrine: No significant differences were observed between groups. (C) Serotonin: A considerable decrease was found in CT+V as compared vs CT+I on day 2. Kruskal-Wallis test, Dunn's post-hoc test. Each dot represents a patient. Median ± 5–95% confidence interval. *P ≤ 0.05. CT= conventional treatment; I= immunomodulator; V= vehicle.

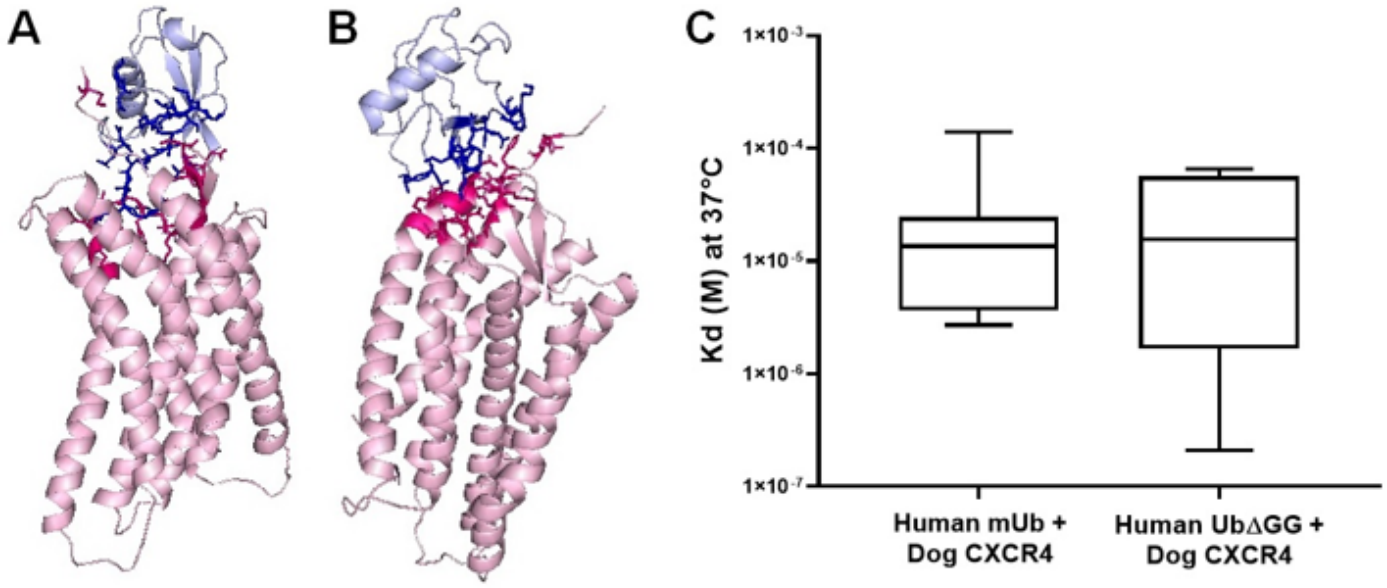

Figure 6

In silico analysis of binding of dog CXCR4 with mUb or UbΔGG. The coupling analysis of dog CXCR4 with mUb (A) or UbΔGG (B) was carried out on the ClusPro 2.0 server. Dog CXCR4 is shown in pink and mUb or UbΔGG in blue. Dark pink or dark blue indicate the interface area between dog CXCR4 with mUb or UbΔGG, respectively. The coupling visualization was done with PyMOL v2.0 (C) and the value of the dissociation constant (Kd) at 37 ºC was assessed from the data obtained with ClusPro and the PRODIGY server. No statistical differences were found between the molecular coupling between the dog CXCR4 and either version of ubiquitin, which indicates that the binding affinity of ubiquitin for the dog CXCR4 is not affected by the deletion of both glycines in the C -terminal. The graph shows mean + range. Using the Mann-Whitney test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.docx
- Additionalfile2.docx
- Additionalfile3.docx